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Fibronectin fragment mediated cartilage chondrolysis. II. Reparative effects of anti-oxidants

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Abstract

In an accompanying manuscript, it was shown that the cartilage chondrolytic activities of fibronectin fragments (Fn-f), which are mediated through catabolic cytokines such as TNF-α, IL-1 and IL-6, could be suppressed by anti-oxidants (AOs). The AOs neutralized reactive oxygen species (ROS) which are known to mediate catabolic cytokine action. The objective in this work was to test whether AOs would promote restoration of proteoglycan (PG) in Fn-f treated cartilage, since under normal culturing conditions, PG is not restored after removal of the Fn-f. Cartilage was first cultured with an amino-terminal 29-kDa Fn-f to cause loss of about half of the total PG and then treated with NAC (1 and 10 mM) or glutathione (10 μM) or DMSO (0.1 or 1%). Treatment with NAC and glutathione maximally caused restoration of PG within 14 days to normal or supernormal levels, while DMSO was less effective. Catalase, but not superoxide dismutase, enhanced PG content to a small but significant extent. The restoration of PG in Fn-f treated cartilage occurred throughout the full depth of the cartilage slices as shown by histochemical analysis. However, removal of the AO allowed a subsequent decrease in PG content suggesting that the AOs had not blocked cytokine expression but had merely suppressed cytokine activities. Addition of NAC to IL-1 treated cartilage promoted a restoration of PG, while addition to chymopapain or trypsin treated cartilage was not very effective, suggesting that the effect of AOs requires a cytokine driven damage system. We conclude that the AOs promote a restoration of PG in the Fn-f treated cartilage by suppressing the effects of catabolic cytokines. The data suggest a potential for AOs in reversing tissue damage caused by cytokines.

Keywords: Fibronectin; Fibronectin fragment; Cartilage; Chondrocyte; Anti-oxidant; Repair; Chondrolysis

1. Introduction

We have reported that specific proteolytic fragments of fibronectin potently cause cartilage chondrolysis, by elevating metalloproteinase (MMP) expression [1] and suppressing synthesis of proteoglycan (PG) [2], which results in rapid rates of PG loss from cartilage tissue in explant cultures [1] and irreversible damage in vitro [2,3]. The most active fragment, an amino-terminal 29-kDa fibronectin fragment (Fn-f) penetrates intact cartilage [4] and may interact indirectly with the fibronectin $\alpha_5\beta_1$ receptor [5] to induce high levels of stromelysin-1 (MMP-3) [6]. Similar fibronectin fragments, when injected into rabbit knee joints, cause over 50% removal of the knee articular cartilage PG within 7 days [7].

The Fn-f exerts its action through TNF-α, IL-1β and IL-6, since neutralizing antibodies to these cytokines suppress MMP-3 release from and reverse PG synthesis suppression in human articular chondrocytes treated with this Fn-f [8]. Since both IL-1 and TNF-α have been reported to operate at least partially through reactive oxygen species (ROS) in cartilage [9,10], we first considered that anti-oxidants (AOs) would suppress Fn-f mediated damage, as defined as loss of cartilage PG, and have reported that AOs can very potently block Fn-f mediated cartilage PG depletion in vitro ([11]; accompanying manuscript). Subsequently it was considered that the inability of Fn-f treated cartilage to restore PG after the Fn-f is removed from the culture [2], may be due to a prolonged expression of

Abbreviations: MMP, metalloproteinase; PG, proteoglycan; Fn-f, a specific amino-terminal 29-kDa heparin-binding fibronectin fragment isolated by thrombin action on human plasma fibronectin; MMP-3, stromelysin-1; ROS, reactive oxygen species; AO, anti-oxidant; DMEM, Dulbecco's modified Eagle's medium; DMB, dimethylmethylene blue; SOD, superoxide dismutase; NAC, *N*-acetylcysteine

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catabolic cytokines or prolonged effects of the cytokines which continue after the Fn-f is removed from the cartilage cultures. This may be due, in turn, to retention of added Fn-f within the tissue or retention within the cartilage tissue of de novo generated cartilage Fn-f or simply due to the cytokine pathways with the unique ability to progress in the absence of the initial stimulant. In any case, this would lead to continual generation of ROS, such as hydrogen peroxide or superoxide anions, which would continue up-regulation of catabolic cytokine effects, such as induction of MMPs and suppression of PG synthesis.

Accordingly, the effects of AOs on restoration of PG in Fn-f treated cartilage tissue have been tested and we report that AOs promote a restoration of PG in Fn-f and IL-1 treated cartilage in vitro but not in cartilage damaged by addition of proteinases. Thus, this repair response of AOs occurs through effects on catabolic cytokines.

2. Materials and methods

2.1. Materials

All common chemicals except where noted, were from Sigma Chemical Co. (St. Louis, MO). Bovine liver SOD (superoxide dismutase) (2500 U/mg protein), catalase, *N*-acetylcysteine, *N*-acetylalanine and reduced glutathione were from Sigma Chemical Co. Protein G-^{125}I (10 μCi/μg) was from ICN Biomedicals (Cosa Mesa, CA). Recombinant human IL-1α was from Genzyme Corp. (Cambridge, MA).

2.2. Isolation of the Fn-f

A well-characterized amino-terminal heparin-binding 29-kDa fibronectin fragment was isolated by sequential cathepsin D and thrombin digests of human plasma fibronectin as described [1]. This is the only fibronectin fragment studied here and is denoted as the Fn-f.

2.3. Explant cartilage cultures

Culturing of articular cartilage slices from adult (18–20 months of age) bovines was performed as described [1–6] in DMEM containing 50 U/ml penicillin/streptomycin with 10% serum/DMEM with 50 to 80 mg cartilage in 2 ml of media. Each datum was a mean and S.D. based on at least three similar tissue culture wells. Each experiment was performed with three different cartilage preparations collected on separate days with similar observations made in each case. Data from a typical cartilage preparation are shown.

2.4. Tests of AOs in facilitating restoration of PG

Cartilage was allowed to equilibrate in 10% serum/DMEM for 1 day, then the culture was adjusted to 0.1 μM Fn-f. Control cultures did not contain the Fn-f. At 7 days, some of the cartilage slices were removed and total PG quantified to ensure that at least 40% of the PG had been depleted. The rest of the cultures treated with the Fn-f were adjusted to media without Fn-f and various concentrations of the AO (typically dissolved at 100 × in DMEM) were added to test for restoration of cartilage PG.

2.5. Assays of PG content of cartilage

The total amount of PG/mg wet wt. cartilage was determined by assays with DMB after treatment of 50–80 mg slices in 1.0 ml of 50 mM phosphate buffer, pH 6, containing 10 mM EDTA and 10 mM cysteine, with 1 mg/ml papain for 8 h at 65°C as originally described [12] with minor modifications [2,5]. The data are reported as μg PG/mg wet weight cartilage based on a mean and S.D. of at least three cartilage samples. The overall variability between similar cartilage culture wells was typically less than 12%.

2.6. Histochemical characterization of tissue

Slices of cartilage were subjected to cryosectioning to obtain full thickness sections of 8 μm. Sections were stained with Safranin-O to reveal matrix PG [13].

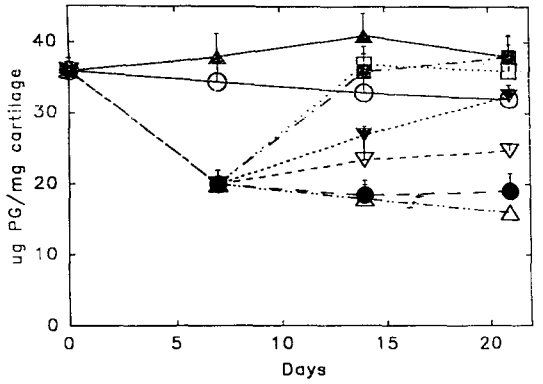

Fig. 1. Effect of NAC on restoration of PG - Cartilage cultures were prepared in 10% serum/DMEM and incubated with 0.1 μM Fn-f for days 0–7. Media were changed every other day with fresh Fn-f added during this period. At day 7, some of the cartilage slices were removed and total PG quantified to ensure that at least 40% of the PG had been removed. The media in the rest of the cultures were then removed and 10% serum/DMEM with test compound added. Cartilage slices were removed at various times and subjected to assays for PG content. Curves shown are untreated control cartilage (○); cartilage treated for days 0–21 with 10 mM NAC (▲); cartilage treated for days 0–7 with 0.1 μM Fn-f, followed by treatment with Fn-f free media (•); cartilage first treated with the Fn-f for days 0–7, followed for days 7–21, by treatment without Fn-f and with 10% serum/DMEM containing 0.01 mM NAC (▽); or 0.1 mM NAC (▼); or 1.0 mM NAC (□) or 10 mM NAC (■) or 1 mM *N*-acetylalanine (△). The media were changed every other day with fresh addition of the AO test compounds.

3. Results

3.1. The AOs, N-acetylcysteine (NAC), glutathione and DMSO promoted a restoration of PG in cartilage temporarily exposed to Fn-f

AOs which had been shown to suppress Fn-f mediated cartilage PG depletion, including, NAC, glutathione and DMSO ([11], accompanying manuscript), were tested for their ability to promote restoration of PG in cartilage first exposed to 0.1 μM Fn-f for 7 days to decrease the PG content. Fig. 1 shows that cartilage first cultured with the Fn-f and subsequently cultured in the absence of the Fn-f did not restore PG; however, addition of NAC promoted a restoration of PG. The concentration required for restoration to normal or supernormal levels was between 1 and 10 mM. A concentration of 0.1 mM caused a restoration to about 75% of normal levels and 0.01 mM caused a significant but incomplete effect. *N*-acetylalanine at 10 mM was not effective, suggesting the importance of the SH group for the activity of NAC. The PG contents of 1 and 10 mM NAC treated cartilage was significantly above control levels as reported in the accompanying manuscript and which we have proposed to be due to suppression of normal rates of PG turnover.

Fig. 2 shows that 10 μM glutathione was totally effective in promoting PG restoration to normal levels and as with NAC, there was an increase to supernormal levels of PG. Fig. 3 shows that 0.1 and 1% DMSO also promoted restoration of PG, however the extent of restoration within 2 wk was less than with NAC or glutathione.

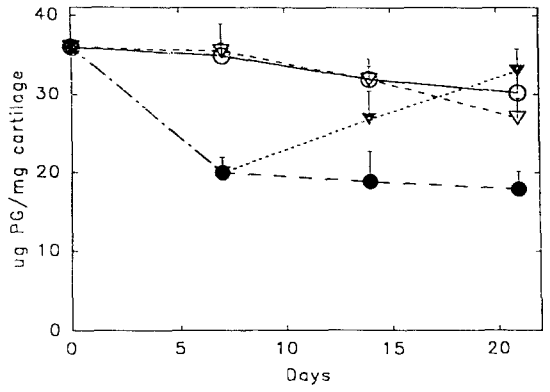

Fig. 2. Effect of glutathione on restoration of PG - Damage or PG depletion conditions were as in Fig. 1, except 10 μM glutathione was used to test PG restoration activity. Media were changed every other day. Cartilage slices were removed at various times and subjected to assays for PG content. Curves shown are control untreated cartilage (○), cartilage treated with 0.1 μM Fn-f for days 0–7, followed by treatment with Fn-f free media for days 7–21 (•), cartilage treated for days 0–21 with 10 μM glutathione (▽); cartilage first treated with the Fn-f for days 0–7, then adjusted to 10 μM glutathione without the Fn-f for days 7–21 (▼).

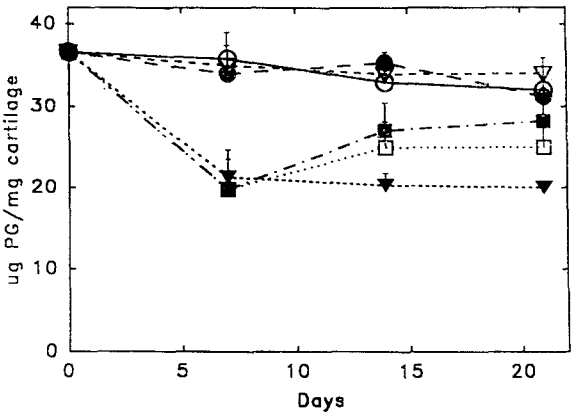

Fig. 3. Effect of DMSO on restoration of PG - Damage or PG depletion conditions were as in Fig. 1, except 1% DMSO was used to test PG restoration activity. Media were changed every other day. Cartilage slices were removed at various times and subjected to assays for PG content. Curves shown are control untreated cartilage (○); cartilage treated with 0.1 μM Fn-f for days 0–7, followed by treatment with Fn-f free media for days 7–21 (▼); cartilage treated for days 0–21 with 0.1% DMSO (•) or with 1% DMSO (▽); cartilage first treated with the Fn-f for days 0–7, then treated with 0.1% DMSO for days 7–21 (□); cartilage first treated with the Fn-f for days 0–7, then treated with 1% DMSO for days 7–21 (■).

3.2. Catalase was only weakly effective on restoration of PG, while SOD was ineffective

The effects of SOD and catalase which would increase or decrease levels of hydrogen peroxide, respectively, were tested. Fig. 4 shows that catalase (highest repair curve) had a slight but significant effect while SOD had no effect on

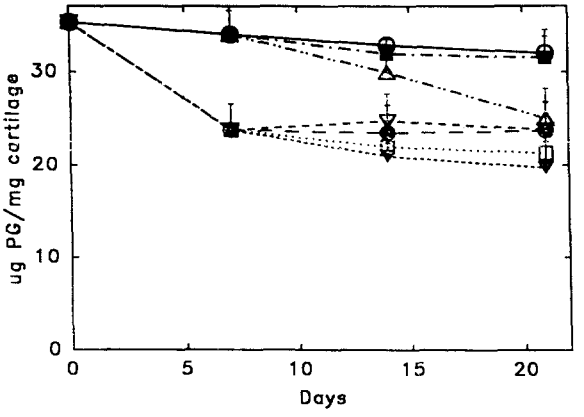

Fig. 4. Effect of SOD and catalase on restoration of PG - Damage or PG depletion conditions were as in Fig. 1 except 5 μg/ml SOD or 5 μg/ml catalase were tested. Media were changed every other day. Cartilage slices were removed at various times and subjected to assays for PG content. The three curves at the top of the figure are untreated control cartilage (○); cartilage treated with catalase alone for days 0–21 (curve is close to control curve) (■); cartilage treated with SOD alone for days 0–21 (△). The bottom family of curves are cartilage treated with 0.1 μM Fn-f for days 0–7, followed by treatment with Fn-f free media for days 7–21 (bottom curve) (▼), cartilage treated with 0.1 μM Fn-f for days 0–7, followed by treatment with 5 μg/ml catalase for days 7–21 (▽); cartilage treated with 0.1 μM Fn-f for days 0–7, followed by treatment with 5 μg/ml SOD for days 7–21 (□); cartilage treated with 0.1 μM Fn-f for days 0–7, followed by treatment with a combination of the enzymes at the same concentrations for days 7–21 (•).

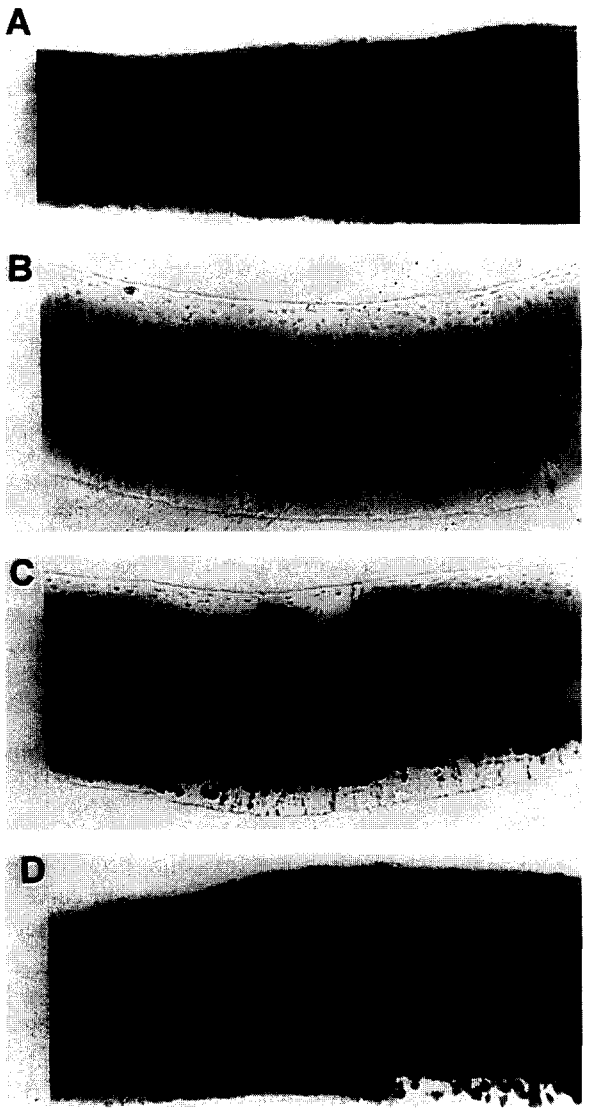

Fig. 5. Histochemical characterization of cartilage treated with the Fn-f and of cartilage treated with the Fn-f, followed by NAC. Cartilage specimens prepared as in Fig. 1 were subjected to cryosectioning followed by staining with safranin O. Shown are untreated control cartilage at 14 days in culture (A); cartilage treated with 0.1 μM Fn-f for 7 days followed by treatment with Fn-f free media for an additional 7 days (B); cartilage treated with 0.1 μM Fn-f for 7 days, followed by treatment with 10 mM NAC for an additional 7 days (C and D). A 10× objective and 2× ocular were used to photograph.

restoration of PG. SOD alone actually caused depletion of PG and when added with catalase reduced the restoration activity of catalase.

3.3. The NAC treated cartilage showed a normal PG staining pattern in full thickness cartilage tissue sections

In order to determine whether the NAC treated cartilage had a normal pattern of PG staining from the superficial zone to the deep zone of cartilage, full thickness sections were prepared by cryosectioning and stained with Safranin-O to visualize PG. Fig. 5A shows control tissue cultured without Fn-f for 14 days. The pattern shows even PG distribution throughout the depth of the full thickness section. Fig. 5B shows cartilage treated with the Fn-f for the first 7 days, then treated with 10% serum/DMEM without the Fn-f for another 7 days. Note the loss of PG in the matrix between the cells which is maintained after the Fn-f was removed. This inability for the PG to return to a normal pattern confirms the lack of reversibility shown earlier with assays of total cartilage PG. Fig. 5C and 5D show similar tissue treated with Fn-f for the first 7 days of culture, then treated with 10 mM NAC-10% serum/DMEM for an additional 7 days. Note that the PG staining pattern between the cells is now as intense as in

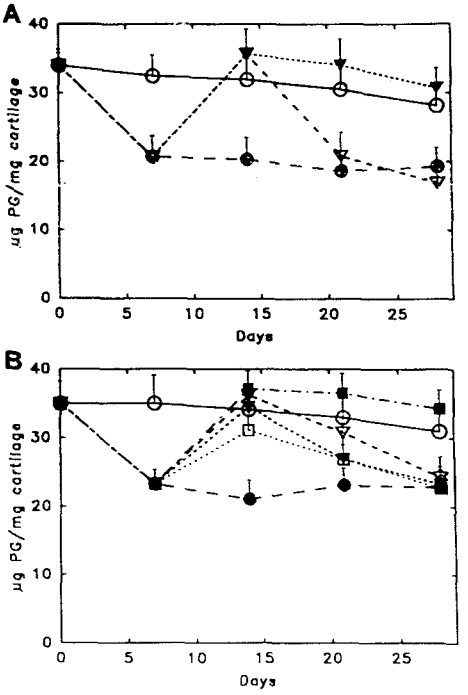

Fig. 6. Effect of removal of anti-oxidant from cartilage first treated with the Fn-f and then with anti-oxidant - For panel (A), cartilage was cultured as described in Fig. 1 with the Fn-f in 10% serum/DMEM for days 0–7, followed by incubation without the Fn-f but with 10 mM NAC for days 7–14 and finally followed by incubation in 10% serum/DMEM without the Fn-f or NAC for days 14–28. Media were changed every other day. Cartilage slices were removed at various times and subjected to assays for PG content. Curves shown are for untreated control cartilage (○); cartilage treated with the Fn-f for days 0–7 only (·); cartilage treated with the Fn-f for days 0–7, followed by treatment with 10 mM NAC for days 7–28 (▼); cartilage treated with the Fn-f for days 0–7 only, followed by treatment with 10 mM NAC for days 7–14 (▽). For panel (B), cartilage was cultured as above, except in 20% serum/DMEM. Curves shown are for untreated control cartilage (○); cartilage treated with the Fn-f for days 0–7 only (·); cartilage treated with the Fn-f for days 0–7, followed by treatment with 10 mM NAC for days 7–28 (■); cartilage treated with the Fn-f for days 0–7, followed by treatment with 10 mM NAC for days 7–14 (▽); cartilage treated with the Fn-f for days 0–7, followed by treatment with 10 μM glutathione for days 7–14 (▼); cartilage treated with the Fn-f for days 0–7, followed by treatment with 1% DMSO for days 7–14 (□).

the control cartilage, although lesser staining is seen on the superficial surface (top) and near the cut edge of the deep zone (bottom).

3.4. Removal of AOs allowed PG depletion to resume

Fig. 6A shows that removal of NAC allowed PG depletion to occur again to the same final extent within 7 days as first observed in the initial incubation with the Fn-f alone. The data also show that inclusion of NAC for the entire culture period of 28 days maintained the PG content at supernormal levels. A higher concentration of serum, 20%, was tested to determine if it would be more effective in facilitating restoration of PG, since it would have a higher amount of serum-derived growth factors, such as IGF-1, and higher levels of proteinase inhibitors. Fig. 6B shows that 10 mM NAC, 10 μM glutathione and 1% DMSO increased the PG content of the Fn-f treated cartilage within 7 days in the presence of 20% serum. However, after removal of the AOs, the PG content quickly decreased within 7 days as also shown in 10% serum.

3.5. NAC also promoted full restoration of PG in IL-1α treated cartilage but not in trypsin or chymopapain treated cartilage

The ability of NAC to promote restoration of PG in cytokine treated cartilage was tested in order to determine if the repair potentials of Fn-f and cytokine treated cartilage are similar, since the damage mechanisms are similar. Cartilage cultured with 50 U/ml recombinant human IL-1α for 7 days showed 38% removal of total PG as shown in

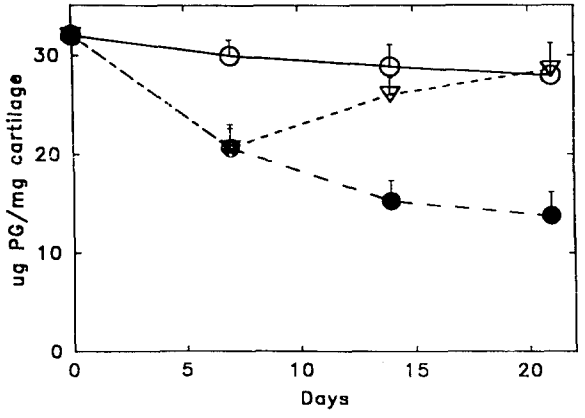

Fig. 7. Effect of NAC on restoration of PG in IL-1α treated cartilage. Cartilage cultures were prepared in 10% serum/DMEM and incubated with 50 U/ml IL-1α for days 0–7. Media were changed every other day and IL-1α added. At day 7, some of the cartilage slices were removed and total PG quantified. The media in the rest of the cultures were then removed and 10% serum/DMEM with 10 mM NAC added. Curves shown are for untreated control cartilage (○); cartilage treated with IL-1α for days 0–7, followed by treatment with IL-1α free media for days 7–21 (•); cartilage treated with IL-1α for days 0–7, followed by treatment with 10 mM NAC in 10% serum/DMEM for days 7–21 (▽).

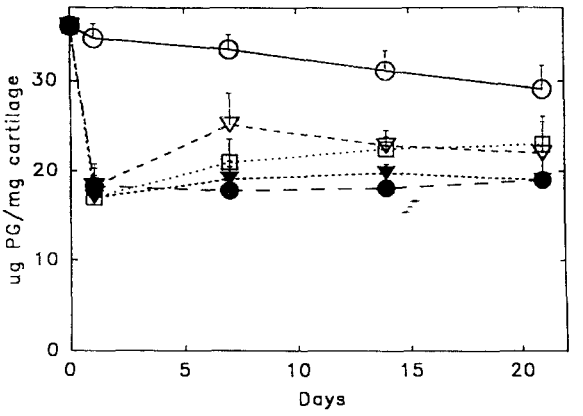

Fig. 8. Effect of NAC on restoration of PG in chymopapain or trypsin treated cartilage. Cartilage cultures were prepared in DMEM and incubated with 0.03 μg/ml chymopapain or with 0.1 μg/ml trypsin for 8 h. The media were then changed and adjusted to 10% serum/DMEM or 10% serum/DMEM containing 10 mM NAC. Curves shown are for untreated control cartilage (○); cartilage treated with chymopapain (•) or trypsin (▼) followed by culturing in 10% serum/DMEM; cartilage treated with chymopapain, followed by treatment with NAC (▽); or cartilage treated with trypsin, followed by treatment with NAC (□).

Fig. 7. After removal of the IL-1α, PG loss continued without NAC but with NAC lost PG was fully restored. The repair potential of cartilage treated with exogenous proteinases rather than cytokines which would induce MMPs was tested also. Fig. 8 shows that the a brief treatment with chymopapain caused over 50% depletion of total PG. Significant PG restoration did not occur subsequent to the damage. However, in cultures treated first with chymopapain and subsequently with NAC, there was a significant increase in PG content by day 12, however the PG content did not increase to control values by day 21. Treatment of cartilage with 0.1 μg/ml trypsin for 2 h in serum-free conditions led also to a 50% loss of PG. Significant PG restoration did not occur subsequent to the removal of the trypsin. Upon treatment of trypsin treated cartilage with NAC, there was only a slight suggestion of an increase in PG content. Therefore, we conclude that NAC was much less effective, within this time frame, on cartilage damaged by the direct action of proteinases.

4. Discussion

As described earlier, Fn-f mediated cartilage chondrolysis is mediated through cytokines, based on the observations that enhanced levels of TNF-α, IL-1 and IL-6 are found in conditioned media and a mixture of neutralizing antibodies to these cytokines totally block PG synthesis suppression and can block up to 83% of the MMP-3 induction by Fn-f [6]. Since we had observed that cartilage treated with the Fn-f does not restore PG after the Fn-f is removed from cultures of bovine articular cartilage tissue

[2,5], we considered that the lack of repair could be due to continuing, albeit perhaps less enhanced levels of catabolic cytokines which continue after the exogenous Fn-f is removed from the cultures. Therefore, we tested AOs which had been shown in the accompanying manuscript and Ref. [8], to promote restoration of lost PG. NAC and reduced glutathione did promote restoration of PG to normal or supernormal levels while DMSO was less effective and tetracycline was not significantly effective. The order of effectiveness of these compounds was the same for their activities in blocking Fn-f activities, which suggests that the target(s) of the AOs in suppressing damage or PG depletion may be similar to the target of the AOs in restoration of PG. Catalase was minimally but significantly effective, suggesting that hydrogen peroxide generated in the Fn-f system is one of the mediators of damage. The inactivity of SOD in promoting restoration of PG is also consistent with a role for hydrogen peroxide in damage since SOD would have enhanced levels of hydrogen peroxide.

The restoration of PG to supernormal levels upon attempted cartilage repair is consistent with data in the accompanying manuscript which shows that the AOs cause a supernormal PG content. This enhanced PG content was associated with a slowing of PG degradation and increase in the half-life of PG in the presence of the AO. Since this suggests that AOs affect normal rates of PG turnover in cartilage, more studies will be required of the long term effects of AOs.

Since the catabolic activities of the Fn-f occur through catabolic cytokines [7], the effectiveness of NAC on IL-1 treated cartilage was tested and found to be maximal. However, NAC was not as effective on cartilage treated with exogenous proteinases. Perhaps a much longer treatment with NAC beyond 30 days might have been more effective, since NAC alone enhanced the PG content of control cartilage and therefore, should have slowed the normal turnover rate of PG enough to enhance reparative processes. One possible explanation for the lesser effect on cartilage treated with exogenous proteinases is that the treatment caused degradation of specific proteins which were required for the restoration of PG, but which were not replaced rapidly enough during the culture period. The cytokine induced MMPs may not have been as destructive. Or perhaps the cytokines directly or indirectly enhanced secondary effects which facilitated repair, such as enhanced release of anabolic factors.

The retention of PG within the cartilage matrix required the continual presence of AOs. This may suggest that the AOs did not suppress synthesis of the catabolic cytokines but instead only suppressed the activities of the cytokines, such as MMP induction and suppression of PG synthesis. Perhaps use of the AO for longer periods, past which catabolic cytokine expression may have decreased to lower levels than may have occurred here, may allow a stable PG content which could be sustained upon removal of the AO.

The effectiveness of the AOs is consistent with the restoration of PG in Fn-f treated cartilage being hampered due to continued expression of catabolic cytokines. Such prolonged cytokine effects after the initial traumatic injury may also hamper cartilage repair in vivo. This first proposal is supported by preliminary observations that show that enhanced levels of IL-1α and MMP-3 continue for up to 21 days in cartilage treated with the Fn-f for 7 days and then allowed to repair (Homandberg et al, unpublished). Explanations for the prolonged enhanced release of cytokines could be that enough of the Fn-f remains trapped in the cartilage during attempted repair or that the damage has generated additional Fn-f from cartilage Fn.

Although repair of cartilage in vivo operates under different constraints than repair in vitro, our results suggest the potential of AOs as therapeutic anti-cytokine drugs, especially in rheumatoid arthritis and osteoarthritis, in which catabolic cytokines play pathologic roles. Further, AOs may have potential in blocking cytokine effects and promoting tissue repair in pathologies of other types of tissue as well.

Acknowledgements

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References

[1] Homandberg, G.A., Meyers, R. and Xie, D.L. (1992) J. Biol. Chem. 267, 3597–3604.
[2] Xie, D.L., Hui, F. and Homandberg, G.A. (1993). Arch. Biochem. Biophys. 307, 110–118.
[3] Homandberg, G.A. and Hui, F. (1994) Arch. Biochem. Biophys. 311, 213–218.
[4] Xie, D.L. and Homandberg, G.A. (1993). Biochim. Biophys. Acta 1182, 189–196.
[5] Homandberg, G.A. and Hui, F. (1994) Arch. Biochem. Biophys. 310, 40–48.
[6] Xie, D.L., Hui, F., Meyers, R. and Homandberg, G.A. (1994) Arch. Biochem. Biophys. 311, 205–212.
[7] Homandberg, G.A., Meyers, R. and Willims, J. (1993) J. Rheumatol. 20, 1378–1382.
[8] Homandberg, G.A. and Hui, F. (1994) Trans. Orthop. Res. Soc. 19, 464a.
[9] Tiku, M.L., Liesch, J.B. and Robertson, F.M. (1990) J. Immunol. 145, 690–696.
[10] Rathawkrishnan, C., Tiku, K., Raghavan, A. and Tiku, M.L. (1992) J. Bone Mineral Res. 7, 1139–1148.
[11] Homandberg, G.A. and Hui, F. (1995) Trans. Orthop Res. Soc. 20, 124a.
[12] Chandrasekhar, A., Easterman, M.A. and Hoffman, H.A. (1987) Anal. Biochem. 161, 103–108.
[13] Rosenberg, L. (1987) J. Bone Joint Surgery 53A, 69–82.